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Glucose repression of lactose/galactose metabolism in *Kluyveromyces lactis* is determined by the concentration of the transcriptional activator LA1C9 (K1GAL4)

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ABSTRACT

In the budding yeast *Kluyveromyces lactis* glucose repression of genes involved in lactose and galactose metabolism is primarily mediated by LAC9 (or K1GAL4) the homologue of the well-known *Saccharomyces cerevisiae* transcriptional activator GAL4. Phenotypic difference in glucose repression existing between natural strains are due to differences in the *LAC9* gene (Breunig, 1989, *Mol.Gen.Genet.* 261, 422–427). Comparison between the *LAC9* alleles of repressible and non-repressible strains revealed that the phenotype is a result of differences in *LAC9* gene expression. A two-basepair alteration in the *LAC9* promoter region produces a promoter-down effect resulting in slightly reduced LAC9 protein levels under all growth conditions tested. In glucose/galactose medium any change in *LAC9* expression drastically affects expression of LAC9 controlled genes e.g. those encoding β-galactosidase or galactokinase revealing a strong dependence of the kinetics of induction on the LAC9 concentration. We propose that in tightly repressible strains the activator concentration drops below a critical threshold that is required for induction to occur. A model is presented to explain how small differences in activator levels are amplified to produce big changes in expression levels of metabolic genes.

INTRODUCTION

Gene regulation mediated by specific transcription factors requires that the activity of these factors responds to the appropriate signals. The question of how this is achieved is a key problem in the molecular analysis of gene regulation at the current state. Early investigations on the prototype transcriptional activator GAL4 have provided a first insight into regulation of a regulatory protein (see (1) for a review). GAL4 induces the genes involved in the metabolism of galactose in *S.cerevisiae* and the GAL4 activity responds to the carbohydrates in the medium. Induction is prevented by the absence of the inducing sugar galactose or by the presence of glucose. Under these growth conditions GAL4 responsive genes are largely inactive. Only the effect of galactose on GAL4 is understood to some extent at the molecular level. It involves the inactivation of the negative regulator GAL80 which in the absence of galactose blocks the activation function. Recent indirect evidence suggests that induction may result from an allosteric transition of the GAL4-GAL80 complex (2, 3). The nature of the galactose induced intracellular signal is unknown. The influence of glucose on GAL4 is even less clear. It has been suggested that glucose may prevent GAL4 from binding to DNA since *in vivo* footprinting data indicated that the GAL4 binding sites in the *GAL1-GAL10* promoter were bound in glycerol grown (derepressed) but not in glucose grown (repressed) cells (4–6). More recently it was shown that the expression of the *GAL4* gene is modestly reduced in glucose as compared to glycerol grown cells and that the level of *GAL4* expression has a strong influence on expression of the GAL4 regulated *GAL1* gene (7, 8). The data indicated that regulation of GAL4 synthesis is an important factor in glucose repression of galactose metabolism. However, a detailed analysis of glucose repression of galactose metabolism has been complicated by the existence of multiple glucose repression pathways in which GAL4 dependent as well as independent mechanisms overlap (8–11). In addition, the inhibitory effect of glucose not only affects the expression of galactose metabolic genes it also acts at the level of galactose uptake through the inactivation of galactose permease (12) and signal generation through the reduction of *GAL3* expression (13–15). A similar complexity exists for glucose repression of other metabolic pathways (reviewed by (16–19)). We have therefore turned to a system which allows to separate the effect of glucose on GAL4 activity from GAL4 independent effects on galactose metabolism.

In the distantly related budding yeast *Kluyveromyces lactis* the regulation of galactose metabolism in general is very similar to that in *S.cerevisiae*. The structure of the *GAL* gene cluster involving *GAL1*, *GAL10* and *GAL7* is conserved, the positioning of the activator binding sites in their control regions is very similar (20), the GAL4 homologue LAC9 binds to the same DNA sequence (21,22) and the two activators are mutually exchangeable (23,24). Coregulated with the *GAL* genes in *K.lactis* are the metabolic genes involved in lactose utilisation, *LAC12* and *LAC4* encoding lactose permease and β-galactosidase,

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Table 1 *Kluyveromyces lactis* strains

strain	parental strain(s)	*LAC9* allele	promoter type[1]	protein sequence[2]	integrative plasmid	Reference
JA6	W600B×SD11.U2	*LAC9-2*	*type 2*	LAC9-L104	none	(22)
JA6/A2*	DL9	*LAC9-2*	*type 2*	LAC9-L104	pLAB92x*Eco*RI/*Pst*I	(32)
JA6/1*	DL9	*LAC9-1*	*type 1*	LAC9-W103	pJ431x*Eco*RI	(28)
JA6/A12*	DL9	*LAC9-12***	*type 1*	LAC9-W103	pLAB91x*Eco*RI/*Pst*I	(32)
JA6/104*	DL9	*LAC9-104***	*type 1*	LAC9-W104	pMa-D104x*Ava*II	(32)
JA6/912	DL9	*LAC9-912***	*type 1*	LAC9-L104	pLAC912x*Ava*II fragment	this work
JA6/2-2*	JA6	*LAC9-2* (2x)	type 2	LAC9-L104	pLI-2x*Bst*EI	(32)
JA6/L92XR	JA6/D9	*LAC9-2XR*	mutant	LAC9-L104	pLAC9-2XRx*Eco*RI/*Hpa*I	(30)
JA6/Z3	JA6/L92XR	*LAC9-2XR*(2x)	mutant	LAC9-L104	pLAC9-2XRx	this work
JA62	JA6/D9	*LAC9-2*	*type 2*	LAC9-L104	pLAC9-2dESx*Bcl*I	this work
JA62/178	JA6/D9	*LAC9-178***	*type 1*	LAC9-L104	pLAC912x*Bcl*I	this work
JA6/D9	JA6	*lac9-2::URA3*	none	none	pDL92x*Eco*RI/*Hpa*I	this work
DL9	JA6	*lac9-1::URA3*	*type 1*	none	pDL9x*Eco*RI/*Hpa*I	(22)
DL9R	JA6	*lac9-1::ura3*	*type 1*	none	FOA^r selection[3]	this work
non-isogenic strains					genotype	
JA6		*LAC9-2*	*type 2*		*α adeA* adeB* ura3 trp1-11*	(22)
NRRL Y-1140		*LAC9-1*	*type 1*	LAC9-W103	**a**	(54)
CBS 2360		not assigned	*type 1*	n.d.	*α*	(54)
W600B		*LAC9-2*	*type 2*	n.d.	*α adeA adeB his*	(44)
SD11.U2=SD12 *LAC9-11*		*type 1*	n.d.	homothallic		(22)

* renamed according to the Kluyveromyces strain nomenclature agreement from JA6-A2, JA6-1, JA6-A12, JA6-104, JA6-2/2 (32)
** alleles have been newly assigned

[1]refers to the sequence between −516 and −42, comp.Fig.3
[2]refers to the amino acid at position 103/104 only
[3]a Ura$^-$ mutant of DL9 obtained by FOA^r selection (55)

respectively. Expression of this latter gene can easily be quantitated allowing to determine LAC9 activity using a natural promoter. Like GAL4, LAC9 responds to the absence of galactose through the action of KlGAL80 (25−27). However, only in some strains it also responds to the presence of glucose. By constructing isogenic strains differing only in the *LAC9* allele we have shown previously that glucose sensitive (*LAC9-2*) and glucose insensitive (*LAC9-1*) *LAC9* variants exist and that glucose repressible or non-repressible β-galactosidase gene expression is determined by the respective *LAC9* allele (28). The term 'glucose repression' refers to the inability of LAC9 to induce the β-galactosidase gene when glucose and galactose (2% each) are simultaneously present in the medium. In this work we have compared the properties of glucose sensitive and insensitive *LAC9* variants. We could demonstrate that the concentration of LAC9 protein in the cell is the crucial factor in determining glucose repression. The relevant parameter that distinguishes the glucose sensitive *LAC9-2* allele from insensitive ones is a promoter mutation which slightly reduces the level of *LAC9* gene expression.

After the experiments shown here were completed similar results were reported by Kuzhandaivelu et al. (29). These authors analysed a repressible strain unrelated to the one used here which contained the same promoter sequence as our repressible strain. They propose that the promoter of repressible strains contains a binding site for a glucose repressor of the *LAC9* gene. We demonstrate that *LAC9* expression is reduced under all growth conditions and suggest that the sequence alteration produces a promoter down mutation which reduces the *LAC9* expression to levels below a critical threshold. The phenomenon of glucose repression of LAC9 controlled genes one of which is *LAC9* itself (30) results from the fact that activation by LAC9 occurs within a sharp boundary only above a certain concentration.

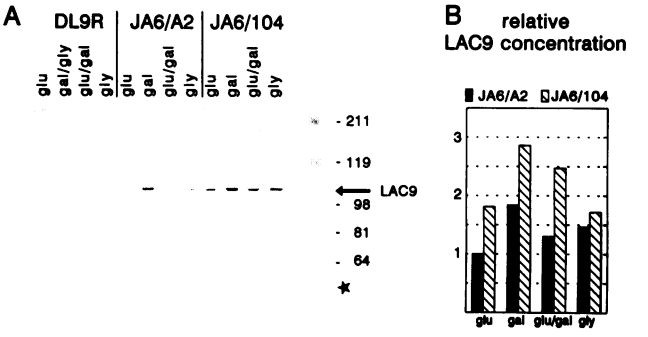

Figure 1. Immunodetection of LAC9 protein. A *lac9* deletion strain DL9R, a glucose repressed strain JA6/A2 and a non-repressed strain JA6/104 were shifted from glucose to media containing either 2% glucose, 2% galactose, 2% glucose + 2% galactose or 3% glycerol as carbon sources. After 5 h protein extracts were prepared, samples of 50 μg of protein were resolved on a 7.5% SDS PAA gel and transferred to a membrane. **A**: The membrane was incubated with an antiserum raised against a GST-LAC9 fusion protein. LAC9 protein was visualised with a secondary antibody linked to alkaline phosphatase. A mixture of prestained proteins (Sigma) was used as a molecular weight standard. **B**: Comparison between JA6/A2 and JA6/104. The LAC9 concentration was quantitated by scanning the membrane shown in A with a laser densitometer. The intensity of the LAC9 signal was normalised to a cross-reacting band (marked *) and concentrations are given relative to JA6/A2 grown on glucose. Maximal deviation between individual experiments was less than 20 % of the mean value.

MATERIALS AND METHODS

Culture conditions

Yeast strains were grown at 30°C in YEP medium (1% yeast extract, 2% bacto peptone). As carbon sources either 2% glucose, 2% galactose, 2% glucose plus 2% galactose or 3% glycerol were added after autoclaving. For the preparation of protein extracts

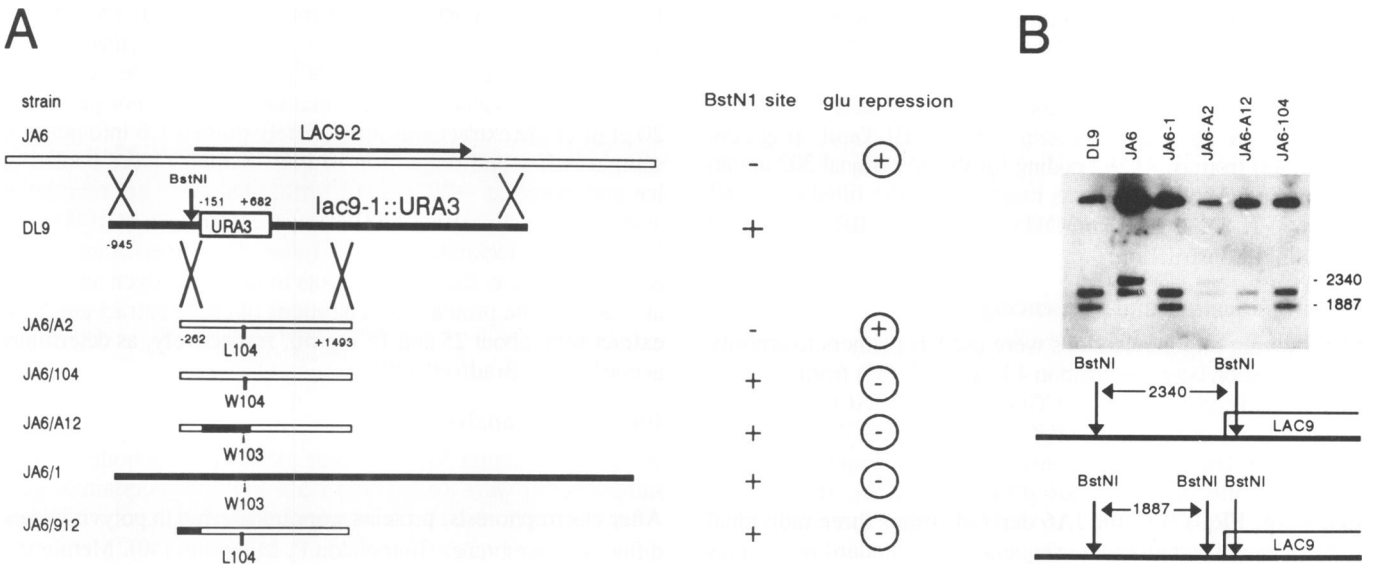

Figure 2. Structure of the *LAC9* locus in JA6 derived strains. **A:** The chromosomal *LAC9* locus of strain JA6 was disrupted by one-step gene replacement to give strain DL9 which in turn was used to restore the *LAC9* gene using the fragments shown below. Open bars indicate *LAC9-2* (JA6 derived) sequences, closed bars *LAC9-1* (Y-1140 derived) sequences. Numbers give the position from the start of translation of *LAC9-1* (35). L104 refers to the leucine residue 104 of the original *LAC9-2* allele which had been implicated in the glucose repression phenotype (33) (see text for details). The corresponding position in *LAC9-1* is W103 due to a reduction in the length of an asparagine stretch further upstream (32). The presence or absence of the *Bst*NI cleavage site indicates whether the site marked in strain DL9 had been retained or was replaced by *LAC9-2* sequences (open bars) in the resulting strains. Glu repression (+) stands for the ability of the respective *LAC9* allele to mediate glucose repression of the LAC9 controlled β-galactosidase gene (*LAC4*) (comp. Fig.4) **B:** Southern blot of the genomic *LAC9* locus. Chromosomal DNA was cleaved by *Bst*NI and hybridised to a *LAC9* specific probe (plasmid pJ431)(23) using a non-radioactive labelling and detection kit (Boehringer, Mannheim, FRG). The structure of the *LAC9-2* (upper line) and *LAC9-1* (bottom line) promoter region is schematically shown below.

strains were grown in glucose containing medium to an OD_{600nm} of 0.6. Cells were washed with prewarmed medium lacking the carbon source and resuspended in the appropriate medium to an OD_{600nm} of 0.1. After 5 h of growth reaching an OD_{600nm} of 0.6−0.8 cultures were cooled on ice and cells were harvested by centrifugation.

Yeast strains and transformation

Kluyveromyces lactis strains are listed in table 1. All JA6 derivatives were obtained by one-step gene displacement (31) in the *lac9* deletion strains DL9 (22) (JA6/x strains) or JA6/D9 (JA62/x series). JA6/D9 does not contain any *LAC9-1* sequences and the deletion is bigger than in DL9 (from −383 to +682) assuring that upon restoration of the *LAC9* gene promoter sequences are provided by the transforming DNA. Restoration of an intact *LAC9* locus was achieved by integrative transformation, selection for growth on lactose and screening for the loss of the Ura^+ phenotype. The correct replacement events were verified by Southern analysis. The control strain JA62 derived from JA6/D9 should be identical to JA6 since all *LAC9* sequences were derived from JA6. In strain JA6/2-2 a second copy of the *LAC9* gene was integrated at the *LAC9* locus as described (32). Strain JA6/Z3 is identical to JA6/2-2 except that both *LAC9* copies carry a linker insertion of the LAC9 binding site in the *LAC9* promoter (30).

For yeast transformation competent cells were prepared as described by Klebe et al. (33) and stored frozen (34). Integrative transformation was carried out with 15 to 30 μg of digested plasmid DNA.

LAC9 nomenclature

The first *LAC9* allele isolated from NRRL Y1140 (23), a non-repressible strain, was designated *LAC9-1* (28), *LAC9-2* was isolated from the glucose repressible strain JA6 (32). Differences between *LAC9-1* and *LAC9-2* reside in the promoter (see Results), in the length of a stretch of asparagine codons (32) and in a Trp>Leu exchange in the Zn^{2+}-binding cluster of the DNA binding domain (32). Additional differences may be present in the region downstream from position 1627. Numbering of the *LAC9* sequence is according to Wray et al. (35) or refers to the distance from the translation initiation codon (ATG = +1).

Plasmids

Plasmid pLAC9-2 contains the *LAC9-2* gene as isolated by integrative cloning from JA6 in a pBR322 derivative (32). In plasmid pLAC9-2dES the *LAC9-1* sequences remaining in pLAC9-2 upstream of the *Sal*I site (Position 374) (35) were removed by cleavage with *Sal*I and *Eco*RI, filling in and religation. In plasmid pLAC912 the *LAC9-2* coding region was fused to the *LAC9-1* promoter in the following way: a genomic fragment amplified by PCR (see below) from strain JA6/104 had been cleaved with *Bgl*II and cloned into pBluescript KS+ (Stratagene, Heidelberg, FRG)×*Bam*HI. The resulting plasmid was cut with *Hin*dIII and a 4.8 kb *Hin*dIII fragment encompassing the *LAC9-2* coding region from pLAC9-2 was inserted. The correct primary structure of the PCR fragment was confirmed by sequencing. The *LAC9* disruption plasmid pDL92 for strain JA6/D9 was derived from pLAC9-2. Sequences between position 560 and 1627 were deleted, a *Hin*dIII linker was inserted instead,

and the *Hin*dIII site downstream of *LAC9* was removed by digestion with *Nco*I and *Bam*HI. The *S.cerevisiae URA3* gene was inserted into the remaining *Hin*dIII site. The plasmid pGEX-L92HX was used for the production of a glutathion S-transferase-LAC9 fusion protein in *E.coli*. A *Hae*III/*Xmn*I fragment (958−1848) from pLAC9-2 coding for the N-terminal 302 amino acids of the LAC9 protein was inserted into the filled-in *Eco*RI site of pGEX-3X (36). *E.coli* DH5αF′ (GIBCO BRL) was used for plasmid construction.

PCR amplification and sequencing

The following oligonucleotides were used as primers to amplify the *LAC9* region between position 431 and 972 (35) from genomic DNA by PCR: oliLAC9P: 5′-CGGAAGATCTCACGGCACGG-GCGTAGC-3′, oliLAC9: 5′-GGCGAATTGGAGGCCCTAC-3′. The resulting fragments were cleaved with *Bgl*II and cloned into the *Bam*HI site of the vector pBluescript KS+ (Stratagene, Heidelberg, FRG). For the JA6 derived strains three individual clones of each fragment were sequenced by standard techniques using the M13 universal primer and a T7 sequencing kit (Pharmacia).

DNA analysis by Southern blotting

DNA isolation, blotting and labelling was performed essentially as described (32).

Isolation of a glutathione S-transferase-LAC9 fusion protein and preparation of antibodies

LAC9 was expressed in *E.coli* as a fusion protein with the carboxyl terminus of *Schistosoma japonicum* glutathion-S-transferase using the expression vector pGEX-3X (36). Chromatography on a glutathion agarose column was used to purify the fusion protein from cell extract of IPTG induced transformants, a modification of the described procedure (36). To separate the fusion protein from contaminating *E.coli* proteins the eluate from a glutathion agarose column was resolved on a preparative 7.5% SDS polyacrylamide gel. After staining the gel with cold 0.1 M KCl the fusion protein was recovered by electroelution using a membrane trap (Schleicher und Schüll)(37) in 25 mM Tris, 192 mM Glycin, 0.1% SDS. A rabbit was immunised by injecting three samples (200 μg each) of fusion protein at four-week intervals (38). The immune serum used in this study was prepared from whole blood collected three weeks after the last booster injection. The specificity of the immune serum for LAC9 was tested by western blotting using a *LAC9* deletion strain and a LAC9 overproducing strain (see also Results). In gel retardation assays the mobility of the LAC9/DNA complex was decreased by immune serum but not by preimmune serum (not shown).

GAL1 specific antibodies were generated in the same way against a GST-GAL1 fusion protein and were generously provided by J.Meyer (Düsseldorf).

Preparation of *K.lactis* protein extracts

Protein extracts used for immunoblotting and gel retardation assays were prepared from 75 OD_{600nm} units of cells. All subsequent steps were carried out at 4°C. Cells were washed once in ice-cold TMEGA300 buffer (0.2 M Tris/HCl pH 7.8, 0.3 M $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 1mM DTT, 10% glycerol) and resuspended in 0.4 ml of the same buffer containing 1 mM PMSF, 4 μM Pepstatin, 4 μM Leupeptin and 14 μg/ml Aprotinin. This suspension was transferred to 1.5 ml Eppendorf tubes containing 0.3 ml of glass beads (ϕ 0.5 mm) and cells were broken using a Braun homogenizer. After centrifugation the supernatant (250 μl) termed crude extract was used for western analysis or gel retardation assays. For the former 20 μl of crude extract was immediately diluted 1:6 into hot SDS sample buffer and boiled for 10 min. Samples were cooled on ice and stored at −20°C until further use. For gel retardation assays the crude extract (200 μl) was centrifuged at 100000×g for 1 h in a Beckman TLA 45 rotor. The supernatant termed S100 extract was frozen in aliquots in liquid nitrogen and stored at −20°C. The protein concentrations of crude extract and S100 extract were about 25 and 15 mg/ml, respectively, as determined according to Bradford (39).

Immunoblot analysis

Samples containing 50 μg of crude extract protein boiled in SDS sample buffer were loaded onto 7.5% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore) (40). Membranes were blocked overnight in PBSTB (PBS containing 3% BSA, 0.1% Tween 20, 0.02% NaN_3) at 4°C and incubated with LAC9 antiserum diluted 1:2000 in PBSTB for 1 h at room temperature. Membranes were washed extensively in PBST (PBS containing 0.3% Tween 20) followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Lab., Avondale, USA) diluted 1:5000 in PBSTB for 1 h. The membranes were washed in PBST followed by a short wash in buffer P (0.1 M Tris/HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) and bound antibodies were visualised in buffer P using the phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate in combination with nitro-blue-tetrazolium chloride.

LAC9 protein concentrations were compared by scanning immunoblots with a Molecular Dynamics Model A300 laser densitometer using Image Quant version 3.15 software. The LAC9 signal was normalised to the signal of a protein with an apparent molecular weight of about 56k. This protein reacted with preimmune- and immune serum and its expression is not regulated by the carbon source.

Gel retardation assays

A 194 bp fragment containing the UASI sequence from the *LAC4* promoter was isolated after *Eco*RI/*Hin*dIII digestion of pUC18-UASI (41) and end-labelled using [α-^{32}P]dATP and Klenow polymerase. Binding reactions were carried out in 15 μl binding buffer (20 mM Hepes/NaOH pH 7.8, 0.1 M NaCl, 10 mM $MgCl_2$, 1 mM Na_2EDTA, 1 mM DTT, BSA 0.2 mg/ml, 10% glycerol) containing 1.2 ng labelled UASI-fragment (9.4 fmol, 1×10^4 cpm), 2.5 μg sonicated calf thymus DNA and 3 or 6 μg S100 protein. Samples of S100 protein were thawed on ice and diluted into cold binding buffer to 1.5 mg/ml immediately before use. Binding reactions were incubated for 20 min at room temperature and loaded on 4% polyacrylamide gels. Gels were run for 2 h at 150V at room temperature in TBE buffer (90 mM Tris, 90mM H_3BO_3, 2.4 mM Na_2EDTA), dried and exposed for autoradiography. Retarded bands were cut out from the dried gel and quantified by Cerenkov counting. Using different amounts of S100 protein the assay was tested to be linear from 1 to at least 8 μg S100 protein.

β-Galactosidase measurements

β-Galactosidase activity was determined at 30°C in crude extracts prepared in β-Gal buffer (5 mM Tris/HCl pH7.5, 10 mM KCl,

5% glycerol) (42) containing 0.1 mM PMSF from 30 OD_{600nm} units of cells. The enzyme assay was carried out in 1 ml β-Gal buffer containing 4 mg o-nitrophenyl-β-D-galactopyranoside (ONPG) and 0.25 mg BSA per ml and the reaction was followed photometrically. The specific enzyme activity was calculated according to Miller (42) except that it was normalised to the amount of protein. The 'Miller-units' are equivalent to mU as used here (μMol of ONP formed per min).

RESULTS

A glucose repressible and a non-repressible strain differ in LAC9 content

The tightly glucose repressible strain JA6 had previously been converted into a non-repressible strain by exchanging its *LAC9* gene (*LAC9-2*) for that of a non-repressible strain NRRL Y1140 (*LAC9-1*) (28). This had been achieved via an intermediate, the *lac9::URA3* disruption strain DL9 (22) (Table 1, comp. also Fig.2A) in which the *LAC9* gene was restored by transformation and selection for growth on lactose. By comparing the two *LAC9* alleles we had detected two alterations in the coding region, a three-basepair insertion that extended a stretch of asparagine codons from eight to nine in *LAC9-2* and a G→T transversion that resulted in a Trp→Leu exchange in the Zn^{2+}-binding cluster of the DNA-binding domain. Introducing both (strain JA6/A12) or the Leu→Trp mutation alone (strain JA6/104) into the *LAC9-2* sequence by *in vitro* mutagenesis and transformation of a suitable DNA fragment into DL9 resulted in a non-repressible strain whereas a control transformation with the same but non-mutated *LAC9-2* fragment gave a strain (JA6/A2) which was tightly glucose repressed like JA6. From these data we had concluded that the Leu→Trp exchange was responsible for the non-repressible phenotype (32).

Since the mutation was located in the DNA-binding domain of the activator we first compared the DNA binding properties of the two protein variants LAC9-L104 and LAC9-W104 from strains JA6/A2 and JA6/104, respectively, using gel-mobility shift assays. However, neither salt dependence, dissociation rates nor equilibrium binding constants showed any significant difference (data not shown). Subsequently, antibodies directed against a GST-LAC9 fusion protein were generated (see materials and methods). The polyclonal antibodies were highly specific for LAC9. On a Western blot of *K.lactis* crude cell extracts (Fig. 1) they reacted with a band of 110k [the expected size for LAC9 is 97.06k (23)] that was absent in extracts of the *lac9* deletion strain DL9R (lanes 1–3) and much stronger in a strain overexpressing LAC9 (not shown).

Again no difference could be detected between LAC9-2 and LAC9-W104 when their mobilities on an SDS gel were compared. However, reproducibly there was a slight increase in the amount of LAC9 in the non-repressible strain JA6/104 (Fig. 1) under all growth conditions which was most pronounced in glucose /galactose medium. Since we had observed before that glucose repression was completely abolished by duplication of the glucose sensitive *LAC9-2* gene (32) this small difference in concentration could be significant.

In both strains the LAC9 concentration depended on the carbon source. It was about 2 to 2.5 fold higher in galactose than in glucose grown cells, in glucose/galactose or glycerol medium an intermediate level was observed.

The glucose sensitive and insensitive *LAC9* alleles differ in the promoter region

An increase in LAC9-W104 concentration could result from a higher stability of this variant due to the Leu→Trp exchange or from a higher level of expression. This latter possibility was carefully examined since additional differences between the *LAC9-2* and *LAC9-1* alleles might exist in the 5′ non-coding region. DL9 (*lac9::URA3*), the parental strain of JA6/104 and JA6/A2 contained sequences derived from the glucose insensitive *LAC9-1* allele which was introduced when the *LAC9* gene was disrupted (22) (Fig. 2A). Since the fragments used to restore an intact *LAC9* locus were derived from *LAC9-2* allelic differences may have been retained between strains JA6/104 and JA6/A2 provided that the heteroduplex formed during homologous recombination was resolved at different positions. To test the identity of the 5′ non-coding region in JA6/104, the control strain JA6/A2 and JA6 we amplified that region between −516 and +26 by PCR. Three independent *BglII* subclones of each of the resulting PCR fragments were sequenced. To our surprise, the JA6/104 derived clones indeed deviated at two positions from

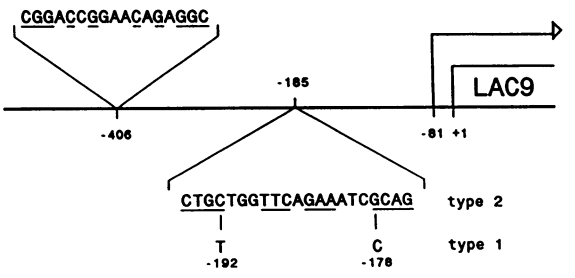

Figure 3. Sequence polymorphism in the *LAC9* promoter region distinguishing glucose repressible and non-repressible strains. The two sequence variants which differ at two positions for glucose repressible strains (type 2, *LAC9-2* allele) and non-repressible strains (type 1, *LAC9-1* allele) are given. At position −406 (from the translation initiation site) the sequence of the LAC9 binding site that confers autoregulation (30) is indicated. Positions related by dyad symmetry are underlined.

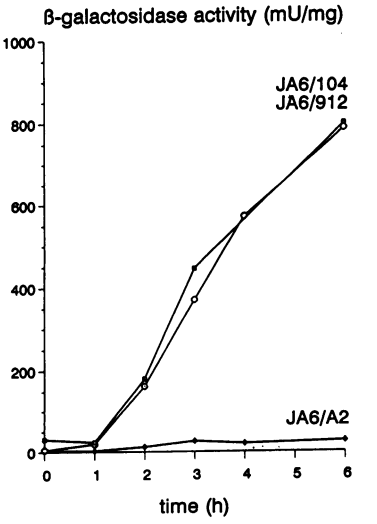

Figure 4. Kinetics of β-galactosidase induction in repressible and non-repressible strains. Strains JA6/A2 (◆), JA6/104 (■) and JA6/912 (○) were shifted from glucose to glucose/galactose (2% each) medium at time 0 and β-galactosidase activity was determined in crude extracts at the indicated time points.

JA6/A2 and JA6: a G→C transition at −178 from the ATG and a C→T transition at −192 (Fig. 3). The sequences of the latter two strains were identical (type 2) whereas the sequence of JA6/104 was identical to the published *LAC9-1* sequence (type 1) (35). Consequently JA6/104 (LAC9-W104) and JA6/A2 (LAC9-L104) not only encoded different LAC9 variants but also contained differences in the upstream region.

One of the two upstream mutations eliminated a *Bst*NI site present in the *LAC9-1* sequence. This offered the possibility to detect the sequence variants by Southern analysis. As shown in Fig.2A and B in all JA6 derived strains (32) the presence of the *Bst*NI site was linked to the glucose non-repressible phenotype. Sequencing confirmed that for the region between −516 and −42 only the two sequence variants existed, type 1 of the glucose insensitive *LAC9-1* allele and type 2 of the glucose sensitive *LAC9-2* allele (Fig. 3). This result strongly suggested that differences in *LAC9* gene expression were responsible for the differences in amount of LAC9 protein in strain JA6/A2 and JA6/104.

The promoter region was also analysed in a few non-isogenic strains (Table 1) after cloning of PCR fragments. In the region between −516 and −42, only the two types of sequences mentioned above were found. The type 1 sequence (Fig. 3) was present in all non-repressible strains whereas the type 2 sequence was found in the tightly repressible strains JA6 and W600B (Table 1).

A small increase in *LAC9* expression is sufficient to alleviate glucose repression

To test whether the promoter mutation was sufficient to determine the glucose repressible phenotype we constructed a strain in which the *LAC9-1* (non-repressible phenotype) promoter was introduced in front of the *LAC9-2* gene (repressible phenotype) (Fig. 2A). The presence of the *Bst*NI site in the genomic DNA of the new strain JA6/912 was verified as above. As shown in Fig. 4 the kinetics of induction of the β-galactosidase gene after shifting the cells from glucose to glucose/galactose medium in this strain JA6/912 was identical to that of the non-repressible strain JA6/104. Thus, the mutations in the promoter region and not the mutation in the Zn-finger were responsible for the loss of LAC9 mediated glucose repression.

To exclude the possibility that again differences in recombination sites introduced additional allelic differences we have also constructed a new *lac9* deletion strain from JA6, JA6/D9 (comp. materials and methods and table 1) which did not contain any *LAC9-1* sequences and in which the promoter region was also deleted. The strains JA62 and JA62/178 resulting from restoration of an intact *LAC9* gene with either a *LAC9-2* fragment (strain JA62) or a type 1 promoter-*LAC9-2* fusion (strain JA62/178) (data not shown) gave exactly the same results as the strains JA6/A2 and JA6/912.

Strains JA6/A2 (repressed phenotype) and JA6/912 (non-repressible) were used to carefully examine the LAC9 concentration in the cell. Since these two strains contain the same LAC9 coding sequence differences in the amount of protein can be attributed directly to differences in gene expression. Western analysis (Fig. 5A) showed a slightly higher LAC9 concentration in JA6/912 than in JA6/A2, very similar to the situation in JA6/104 (Fig. 1). To further support the significance of this small difference we also determined the LAC9 concentration by an independent method. In this case the DNA-binding activity was quantitated by gel-retardation. The relative binding activities in crude extracts (Fig. 5B) showed a good correlation with the Western analysis (Fig. 5A) and were about 1.5-fold higher in strain JA6/912 than in JA6/A2. Thus, a very small increase in LAC9 concentration seems to be sufficient to allow for induction in the presence of glucose.

Inducibility of the *LAC/GAL* regulon is determined by the LAC9 concentration

To confirm this conclusion we analysed the *LAC9* content in a strain containing two *LAC9* genes, JA6/2-2. As shown before glucose had almost no effect on the rate of induction of the β-galactosidase gene (32) in this strain which contains two *LAC9-2* copies tandemly integrated at the *LAC9* locus. Quantitation of LAC9 levels gave two-fold higher values in glucose, in agreement with the gene dosage (Table 2). In galactose and glucose/galactose, however, the LAC9 concentration was higher than expected from the copy number. As detailed elsewhere (30) this overexpression is due to positive autoregulation mediated by the LAC9 binding site located at position −406 of the *LAC9* promoter (Fig. 3). To obtain a more subtle increase in *LAC9* levels, similar to the situation in JA6/912, this binding site was mutated by linker insertion in both gene copies of JA6/2-2 resulting in strain JA6/Z3. In glucose/galactose the LAC9 level of this strain was intermediate between JA6 and JA6/912 (Table 2). The four strains JA6, JA6/Z3, JA6/912 and JA6/2-2 thus represent a series of four isogenic strains with increasing concentrations of LAC9 in which expression of two *LAC9* controlled genes *GAL1* and *LAC4* was determined (Table 2). Quantification of the *GAL1* and *LAC4* gene products by immunoblotting and enzyme assays, respectively, showed that under each of the growth conditions tested the protein levels correlated with the relative LAC9 concentrations. In glucose/galactose medium the *LAC/GAL* regulon responded particularly sensitive to the activator concentration. It was therefore crucial to determine all protein concentrations from the same culture. Then, even the very small difference between JA6/Z3 and JA6/912 was reflected in *GAL1* and *LAC4* gene expression. Both strains could still be induced in glucose/galactose although much less efficiently than JA6/2-2.

The LAC9 content of JA6/Z3 and JA6/2-2 differed by a factor of three. In galactose they were induced to almost the same extent indicating that expression of *GAL1* and *LAC4* in JA6/2-2 is no longer limited by LAC9. In glucose/galactose, however, induction was largely inhibited in JA6/Z3 and only slightly in JA6/2-2 (comp. gal and glu/gal values in Table 2). Thus glucose repression could almost completely be restored when the *LAC9* promoter was manipulated other than in the region that distinguishes type 1 and type 2 promoters. Most likely the type 2 sequence functions in glucose repression solely through a reduction in *LAC9* promoter strength and any other promoter-down mutation has a similar effect. We propose that for induction in glucose/galactose the LAC9 activator has to reach a critical concentration. When the *LAC9* gene is present in a single copy its expression level with the type 2 promoter is just below with the type 1 promoter just above that critical threshold. All the analyses presented above were done in the genetic background of JA6 which is exceptional among *K.lactis* strains by showing tight repression of the β-galactosidase gene. The fact that glucose repression in this strain can be overcome by increasing the LAC9 concentration does not necessarily imply that the lack of repression in non-isogenic strains is also due to elevated levels of LAC9. We have therefore analysed some additional strains.

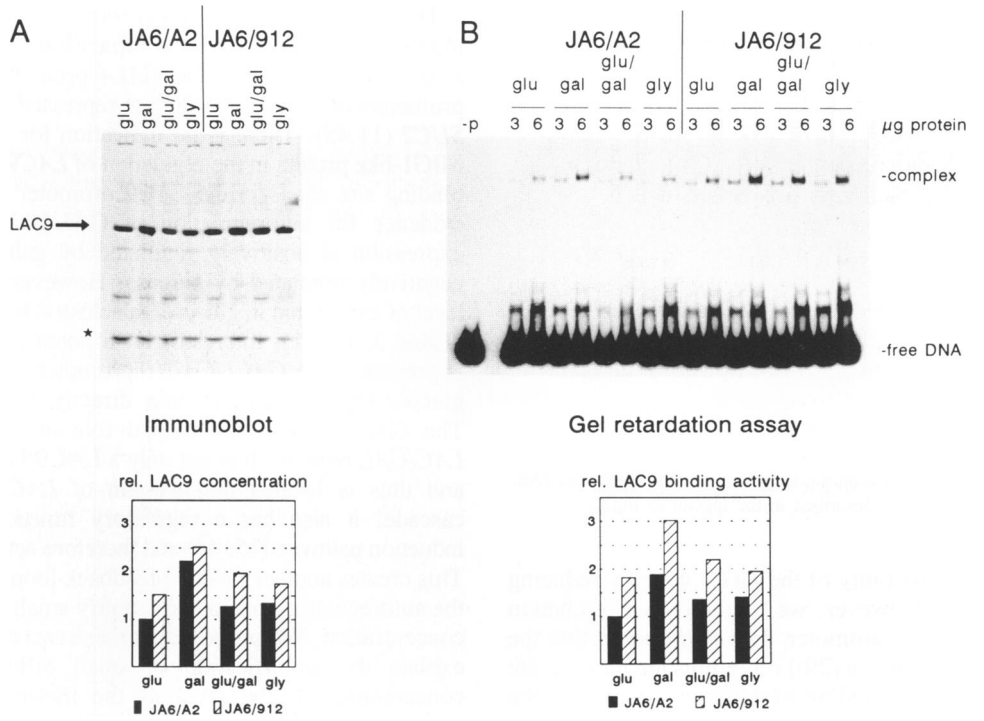

Figure 5. Quantification of LAC9 concentration in JA6/A2 (glucose-repressed) and JA6/912 (non-repressed) by immunoblotting and gel retardation assay. These strains are isogenic except for a two-basepair alteration in the *LAC9* promoter. **A**: Immunoblot was carried out and quantified essentially as described in Fig. 1. **B**: LAC9 binding activity was determined by gel retardation assay. 3 and 6 μg of S100 protein were incubated with 2.5 μg of calf thymus DNA and 1.2 ng of a labelled fragment (9.4 fmol, 1×10^5 cpm) containing the UASI sequence of the *LAC4* promoter in a total volume of 15 μl. Binding reaction were incubated for 20 min at room temperature and loaded onto a 4% polyacrylamide gel. After electrophoresis at 150V in TBE buffer gels were dried and autoradiographed. LAC9 binding activity was quantitated by Cerenkov counting of the retarded bands cut out from the dried gel and is given in relation to JA6/A2 grown in glucose.

Table 2. Expression of *LAC4* and *GAL1* in strains containing different concentrations of LAC9 protein

	JA6			JA6/Z3			JA6/912			JA6-2/2		
	glu	gal	glu/gal	glu	gal	glu/gal	glu	gal	glu/gal	glu	gal	glu/gal
LAC9	1.00	1.50	1.04	1.32	3.81	1.53	1.63	2.78	1.96	1.94	8.99	5.03
LAC4	1.1	3257	7.6	1.8	4259	114	1.8	3292	375	6.0	5631	2519
GAL1	<0.2	40.9	0.53	<0.2	41.3	2.8	<0.2	42.2	9.1	<0.2	48.9	24.6

Concentrations of LAC9 and GAL1 were measured by densitometric scanning of immunoblots. LAC9 concentrations are given in relation to JA6 grown in glucose and GAL1 concentrations are given in arbitrary units. Expression of *LAC4* was quantitated by measuring β-galactosidase activity and is given in mU/mg.

W600B is tightly glucose repressed like JA6, in strain Y1140 a transient effect of glucose is seen, resulting in a short delay in induction whereas almost no inhibitory effect of glucose exists in CBS2360 and SD11. As shown in Fig.6 in all repressible strains the amount of LAC9 is lower than in non-repressible or weakly repressible ones. Again the degree of repression in the order W600B/JA6 > Y1140 > CBS2360 (28) correlates with the relative LAC9 levels extending our conclusions to non-isogenic *K.lactis* strains that the concentration of the activator is the major determinant for induction under these growth conditions.

DISCUSSION

Both GAL4 and its *K.lactis* homologue LAC9 are known targets of glucose repression. We have made use of the existence of natural glucose sensitive and insensitive *LAC9* alleles to analyse the inhibitory effect of glucose on this transcriptional activator. The results presented demonstrate that although multiple differences exist between these alleles it is the difference in the *LAC9* promoter region which is responsible for glucose repression of the *LAC9* controlled gene *LAC4*. A two-basepair alteration at positions −178 and −192 from the ATG is sufficient to convert a glucose repressible strain into a non-repressible one. The type 2 sequence (Fig.3) linked to the strongly glucose repressible phenotype in the *K.lactis* strains examined in this work was also found in another repressible strain Y1118 and was shown to confer glucose repression by the group of Dickson (29). Whereas these authors have compared *LAC9* steady-state mRNA levels of repressible and non-repressible strains in glucose/galactose medium we have determined the LAC9 protein concentration and the LAC9 specific DNA-binding activity under various growth conditions. We agree with the primary conclusion of Kuzhandaivelu et al. (29): The type 2 promoter sequence

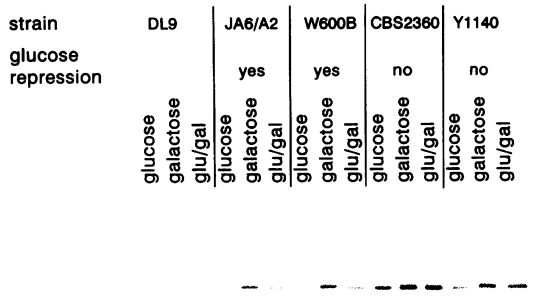

Figure 6. Comparison of LAC9 concentration in strains non-isogenic to JA6. Immunoblotting was carried out as described in the legend to Fig.1.

introduces glucose repressibility of the *LAC4* gene by reducing *LAC9* gene expression. However, we disagree on the function of this region in the *LAC9* promoter. It was proposed that the type 2 sequence [named URS in (29)] represents the binding site for a glucose repressor the binding of which is affected by the mutation. We could show that *LAC9* gene expression is influenced by the two-basepair alteration no matter whether glucose is present in the medium or not. In addition, we have shown that glucose repression was also affected by manipulating the *LAC9* promoter other than through the so-called URS. We therefore propose that the type 2 sequence is not directly involved in mediating glucose repression of the *LAC9* gene but affects *LAC9* promoter activity under all growth conditions. To explain its specific influence on glucose repression we suggest the following model: Glucose repression of the LAC9 controlled genes is a manifestation of the activator's inability to induce transcription in glucose/galactose when its concentration is too low. Below a critical threshold no induction takes place as was observed in strains containing a single *LAC9* gene with the type 2 promoter. Above that threshold the rate of induction crucially depends on the activator concentration. A two-fold difference in *LAC9* expression levels had a drastic influence not only on expression of the β-galactosidase gene *LAC4* but also of *GAL1* and probably all other LAC9 controlled genes.

One of these genes is *LAC9* itself (30). The autoregulatory induction of *LAC9* represents a positive feedback loop providing a means to amplify small differences in activator concentration. The activator binding site in the *LAC9* promoter is a low affinity site (30) and since LAC9 protein is limiting even under inducing conditions (30,41) its occupancy should crucially depend on the concentration of LAC9 protein.

Autoregulation may also explain glucose repression of the *LAC9* gene. Since for the metabolic genes induction in glucose/galactose requires that the activator level reaches a critical concentration the same may be true for the *LAC9* gene itself. In that case glucose repression of *LAC9* gene expression could solely be mediated by LAC9.

It has to be emphasised that a low activator concentration is not sufficient to block induction. Higher activator levels (or a higher specific activity) are required for induction in the presence of glucose (30). Whether that relates to glucose specific changes in the state of the activator as reported for GAL4 (43,44) or to alternate structures of the target genes (5, 6) or both remains to be shown.

The homologous gene in *S.cerevisiae*, *GAL4* is also a glucose repressed gene (7,11). *GAL4* repression is mediated by MIG1, a protein that binds to the *GAL4* promoter as well as to the promoters of some other glucose repressed genes like *GAL1* and *SUC2* (11,45). There is no indication for an involvement of a MIG1-like protein in the regulation of *LAC9*, at least no potential binding site exists in the *LAC9* promoter whereas there is no evidence for autoregulation of GAL4. It seems that *LAC9* expression is positively regulated by galactose and *GAL4* is negatively regulated by glucose. However, for both genes the level of expression in glucose/galactose is lower than in galactose (Table 2, ref.11). Interestingly, a potential MIG1 binding site is present in the *GAL1-GAL10* promoter of *K.lactis* such that a glucose repression signal may directly be sensed at that level. The *GAL1* gene has a crucial role in the regulation of the *LAC/GAL* regulon. It is not only a LAC9/GAL4 controlled gene and thus is located downstream of *LAC9* in the regulatory cascade, it also has a regulatory function required for the induction pathway (15, 46) and therefore acts upstream of *LAC9*. This creates another positive feedback loop which in addition to the autoregulatory one could amplify small differences in *LAC9* concentration. We propose that these two control circuits could explain the amplification of small differences in activator concentration at the level of the metabolic genes and that cooperativity between multiple LAC9 molecules as was proposed (29) is not required although it may contribute to the observed effects. In line with this model differences in glucose repression between JA6 and JA6/912 also exist for a reporter gene controlled by a single LAC9 binding site (47).

Probably glucose repression mediated by GAL4 in *S.cerevisiae* in principle is similar to the mechanism proposed here. In *S.cerevisiae* as in *K.lactis* induction by GAL4 critically depends on the concentration of the activator and the regulation of the *GAL4* gene plays a crucial role in glucose repression (7, 8). However, there are also a few important differences, one of them concerning the role of GAL80. In *S.cerevisiae* the influence of small variations in activator concentration on glucose repression were only seen in a *gal80*$^-$ background (8) whereas the effect in *K.lactis* was shown in a *GAL80* wildtype strain. According to our model the specific activity of the LAC9 activator in glucose/galactose just had to be closer to the critical threshold than that of GAL4. In line with this proposal, a more drastic overproduction of GAL4 affected glucose repression in a *GAL80* background of *S.cerevisiae* (48).

GAL80, the negative regulator of the induction pathway could fulfil the role of limiting the positive feedback loops. The *K.lactis GAL80* gene has recently been cloned in our lab. Disrupting the gene revealed that in contrast to *S.cerevisiae* (8,49,50) glucose repression was almost completely lost in a *gal80* mutant of JA6 (27). Due to the autoregulatory induction of the *LAC9* gene the mutation in *K.lactis* not only affects the activity of *LAC9* but also its concentration (27) and therefore further amplifies the induction pathway. It has been studied extensively in the regulation of the life cycle of phage lambda (51) and other prokaryotic model systems how small differences in the concentration of a regulatory molecule can result in an alternate pattern of gene expression. Other examples emerge from developmental biology where concentration gradients of morphogens or other regulators play an important role in determining the future fate of a cell (52,53). However, most studies on gene regulation in eukaryotes reveal that an awareness of the significance of small differences in the abundance or activity of a regulatory molecule is only beginning to emerge.

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